DICK HEINEGARD

PROTEOGLYCANS FROM CARTILAGE AND OTHER CONNECTIVE TISSUES

LUND 1974

From the Department of Physiological Chemistry,
University of Lund, Lund, Sweden.

PROTEOGLYCANS FROM CARTILAGE AND OTHER CONNECTIVE TISSUES

by

Dick Heinegård

Lund 1974

UNIV.
LEIDEN
BIBL.

This dissertation is based on the following communications:

I. Dick Heinegård
Extraction, fractionation and characterization of proteoglycans from bovine tracheal cartilage
Biochim. Biophys. Acta 285 (1972) 181-192

II. Dick Heinegård
Hyaluronidase digestion and alkaline treatment of bovine tracheal cartilage proteoglycans.
Isolation and characterisation of different keratan sulfate proteins
Biochim. Biophys. Acta 285 (1972) 193-207

III. Dick Heinegård and Vincent C. Hascall
Characterization of chondroitin sulfate isolated from trypsin-chymotrypsin digests of cartilage proteoglycans

IV. Vincent C. Hascall and Dick Heinegård
Aggregation of cartilage proteoglycans:
I. The role of hyaluronic acid
J. Biol. Chem., In press

V. Vincent C. Hascall and Dick Heinegård
Aggregation of cartilage proteoglycans:
II. Oligosaccharide competitors of the proteoglycan - hyaluronic acid interaction
J. Biol. Chem., In press

VI. Dick Heinegård and Vincent C. Hascall
Aggregation of cartilage proteoglycans:
III. Characteristics of the proteins isolated from trypsin digests of aggregates
J. Biol. Chem., In press

VII. C.A. Antonopoulos, Inge Axelsson, Dick Heinegård and Sven Gardell
Extraction and purification of proteoglycans from various types of connective tissue
Biochim. Biophys. Acta 338 (1974) 108-119

The articles will be referred to in the text by their Roman numerals.

CONTENTS

INTRODUCTION.....5

PREVIOUS INVESTIGATIONS.....5

PRESENT INVESTIGATION.....14

Methodology.....14

Isolation and Characterization of proteoglycans from tracheal cartilage.....15

Purification of link proteins.....17

The proteoglycan protein core.....18

Distribution of chondroitin sulfate side chains in proteoglycans.....21

Aggregation of proteoglycans.....24

Characteristics of aggregates from nasal and tracheal cartilage.....24

The role of hyaluronic acid in aggregation.....26

Specificity of the interactions.....28

Characterization of the structures interacting with hyaluronic acid.....30

Proteoglycans from aorta, cornea and sclera. Preparation and characterization.....32

GENERAL CONCLUSIONS.....35

TENTATIVE MODEL FOR THE STRUCTURE OF PROTEOGLYCAN AGGREGATE.....37

ACKNOWLEDGEMENTS.....38

REFERENCES.....39

INTRODUCTION

Connective tissues in general are characterized by a low density of cells and an abundance of extracellular material composed of fibers and an amorfous gel, earlier called the ground substance. In hyaline cartilage less than 10 percent of the tissue dry weight is cellular material, about half of the remainder is fibers, mostly collagen, and the rest is ground substance. The collagen forms a network of interlaced fibers of different diameters. Within this network the cells and the amorfous gel are located. The gel is considered to be of major importance for the properties and biological function of the cartilage. It e.g. contributes to the resilience of the tissue and is necessary for the function of cartilage as a shock absorber. Thus, in order to better understand the behavior of cartilage in normal and pathological conditions, it is a problem of great biological significans to reveal what molecular organization may determine the properties of the extracellular material in cartilage. It is the purpose of this treatise to present some contributions to the solution of the problem.

PREVIOUS INVESTIGATIONS

A century ago the first experiments were undertaken in order to shed some light on the chemical composition and structure of the "ground substance". In 1838 Mulder (1) discovered that by acid hydrolysis, sulfate could be liberated from a substance called chondrin, which could be isolated from cartilage.

About fifty years later, in 1884, Krukenberg (2) extracted cartilage with alkali. From the extract he precipitated with ethanol a substance acidic in nature, and with a composition on elementary analysis similar to that of, what today is called chondroitin sulfate.

Further progress was made by Mörner (3) in an extensive study of the structure and chemistry of cartilage. After several days of autolysis, he iso-

lated a sulfate-containing preparation called chondromucoid. This material had a much higher content of nitrogen, than the corresponding preparation that he prepared from alkaline extracts.

Schmiedenberg (4) created the name chondroitin sulfuric acid and gave it to the substance isolated from alkaline extracts of cartilage. He also degraded the material by acid hydrolysis and concluded that the substance contained hexuronic acid and hexosamine.

The next big step forward came through the work of Levene and his collaborators (for reference see 5), who showed that chondroitin sulfate was composed of sulfate, galactosamine (at that time called chondrosamine) and glucuronic acid.

It was not until the middle of the 1940-ties that the structural work on the chondroitin sulfate as a polysaccharide began, and thanks mainly to the work of Meyer and collaborators (6, 7, 8, 9) the detailed structure of the chondroitin sulfate was revealed during the 1950-ties. It was shown that the polysaccharide consists of 1-4 linked repeating disaccharide units built from d-glucuronic acid linked via a β 1-3 linkage to N-acetyl-galactosamine. The hexosamine is esterified with sulfate either at position 4 or 6 (10). The number of repeating units is usually less than 50 and the molecular weight is just above 20,000 (11, 12, 13, 14) with a large polydispersity (13). One such molecule would contain in the order of 100 negatively charged groups.

Among the glycosaminoglycans[1], although not present in hyaline cartilage but relevant for the discussions in this treatise, is also dermatan sulfate originally called chondroitin sulfate B and composed of disaccharides of

---

1) Meyer (15) called substances like chondroitin sulfate acidic mucopolysaccharides. In an attempt to create a more rational nomenclature Balazs and Jeanloz (16) introduced the name glycosaminoglycans for polysaccharides containing hexosamines.

N-acetyl-galactosamine and iduronic acid with varying numbers of the iduronic acid residues exchanged by glucuronic acid. Hyaluronic acid is a non-sulfated glycosaminoglycan built from a large number of N-acetyl-glucosamine-glucuronic acid disaccharide units.

Another polysaccharide component of cartilage that usually constitute less than 10 % of the glycosaminoglycans is keratan sulfate, first detected in cornea by Meyer and coworkers (17). This molecule consists primarily of repeating units of a disaccharide of N-acetyl-glucosamine linked via a 1-3 linkage to galactose (18). The hexosamines are esterified with a sulfate group at carbon 6. The number of repeating disaccharide units in keratan sulfate appears to be much lower than that of chondroitin sulfate, since the molecular weight is lower (19).

In 1924 Hammarsten (20) had shown that nucleic acid could be extracted in good yield from the tissues by weak salt solutions. Since chondroitin sulfate like the nucleic acids is a polyanion, Jorpes in 1929 (21) thought that the polysaccharide could also be extracted by weak salt solutions. He found, however, that only a few percent of the polysaccharides were salt extractable and concluded that treatment with 0.5 M alkali was necessary to reach a quantitative recovery of the chondroitin sulfate. In spite of the fact that strong alkali was needed to extract the polysaccharide, it was for a long time considered that chondroitin sulfate was bound to the proteins in the tissue by salt linkages (22), in analogy to the nucleic acids.

Based on the observation that the protein-polysaccharide "complex" was stable over a large pH-range Partridge (23), however, concluded that a salt linkage between the acid polysaccharides and proteins was less likely. Heating the preparation of "complex" was found to cause a rapid reduction in viscosity (23).

An important contribution to the understanding of the structure of the

ground substance was made by Muir in 1968 (24). She extracted a chondroitin sulfate-protein complex using 10 % $CaCl_2$ in water and subsequently purified the polyanions by precipitation with the dye 5-amino-acridine. Papain digestion of the material followed by precipitation of the chondroitin sulfate yielded a preparation that contained as much as half of the serine in the original preparation, while the contents of other amino acids were low. The high content of serine in chondroitin sulfate, even after extensive proteolysis and purification, offered strong support for the idea that the linkage between the polysaccharide and protein was of covalent nature. Since peptides could be liberated from the chondroitin sulfate by alkaline treatment she proposed that the linkage to protein was an ester bond (24).

The work of Gregory et al. (25) provided strong indirect evidence that the linkage was a glycosidic linkage from galactose or xylose to serine. In the crucial experiments by Anderson et al. (26) it was shown that when alkaline treatment was used to remove the peptide from the chondroitin sulfate the serine was converted to dehydroalanine. Obviously a β-elimination reaction had taken place and consequently they concluded that serine was linked via an O-glycosidic bond to the chondroitin sulfate.

Through the elegant work of Rodén and coworkers (27, 28, 29, 30) the structure of the linkage region was established to be serine-xylose-galactose-galactose-glucuronic acid.

In contrast work on keratan sulfate peptides have failed to conclusively establish a specific sequence of monosaccharides in the linkage region to protein, although the work of Anderson et al. (31) showed that a galactosamine residue in the keratan sulfate molecule is linked to protein via an O-glycosidic bond to serine or threonine.

By prolonged water extraction of cartilage Shatton and Schubert (32) isolated a chondroitin sulfate-protein (protein-polysaccharide)[1] which was purified

---

1) The term proteinpolysaccharide was used for the complex of glycosaminoglycan and protein. A more recently introduced term for such complexes is proteoglycan.

to contain no free chondroitin sulfate and no collagen.

A model for the structure of protein-polysaccharides that in essence is similar to the one currently accepted was proposed by Mathews and Lozaityte (33). They used a protein-polysaccharide preparation that contained about 16 % protein to determine physical properties before and after degradation of the chondroitin sulfate side chains with hyaluronidase. Light scattering measurements gave a M_w value for intact protein-polysaccharides of 4×10^6. From their data they concluded that the molecules were rod-shaped with dimensions of a central protein core of 3700 Å and that some 60 chondroitin sulfate chains, each attached by one or more bonds to the protein core, radiated out from the core to produce a comblike structure. They also proposed that aggregates of M_w 50×10^6 could be formed by association of basic units. The model proposed by these authors was supported by the investigations of Partridge and collaborators (34, 35), who also suggested that keratan sulfate was linked to the same protein core as chondroitin sulfate and that each chondroitin sulfate chain had only one attachment point to protein.

The early investigations on protein-polysaccharide structure, however, all suffered from the same objection. Were the protein-polysaccharides representative of the native molecules in cartilage or were they degradation products obtained by proteolysis during the prolonged extraction with water or weak salt solutions? Proteolytic enzymes are known to be present in cartilage (34, 36, 37) and autolysis had for long been used to liberate the glycosaminoglycans.

A great improvement was the new methods introduced by Malawista and Schubert (38). In order to improve the yield of protein-polysaccharides they used high speed homogenisation of the cartilage in water at low temperature for short time intervals, thereby also decreasing the risk for degradation by proteolysis. The protein-polysaccharides in the extracts were then precipitated with ethanol and the yield was about 80 % of the total amount of protein-polysaccharide in the tissue. In a series of works Schubert and

coworkers (39, 40, 41) showed that by ultracentrifugation they could separate the protein-polysaccharides into several subfractions, called PPH and PPL 2, 3, 4, 5 and 6, with different composition and with different molecular sizes. For instance PPL-3 showed one component in the analytical ultracentrifuge, while PPL-4 showed two. Thus cartilage protein-polysaccharide complexes appeared to show both polydispersity and heterogeneity.

Several authors have since used the Malawista and Schubert technique (38) to isolate protein-polysaccharides. Buddecke et al. (11) purified a protein-polysaccharide using CPC-precipitation and obtained a preparation that contained 16 % protein and that had an S_o value of about 10S and a calculated weight of 500,000. These authors suggested that each core contained 20-30 chondroitin sulfate chains. In a later work (42) the M_w was found to be 3×10^6 and the length of the core 3000 A.

Luscombe and Phelps (43) prepared a PPL with a molecular weight of $3.2 - 5.8 \times 10^6$, and a radius of gyration of 1390 A. They also demonstrated a dependance of relative viscosity on ionic strength (below 0.2 M salt) and pH. They suggested that the protein-polysaccharide had a structure of a random coil of an overall spherical shape.

Indirect evidence was presented using different protein-polysaccharide preparations that keratan sulfate and chondroitin sulfate were linked to the same protein core (44, 45).

Muir and Jacobs (46) used mild extraction methods in weak salt to isolate a small molecular weight protein-polysaccharide with a low protein content and a high chondroitin sulfate to keratan sulfate ratio. These authors also isolated a large molecular weight fraction. Their results indicate heterogeneity of the protein-polysaccharide. Similar results have since been obtained by Tsiganos and Muir (47).

Based on studies with chemical degradation, Serafini-Fracassini et al. (48) suggested that the protein-polysaccharides were built from subunits of two

chondroitin sulfate chains joined by a short peptide.
Serafini-Fracassini et al. (49) also demonstrated the presence of proteolytic activity in PPL fractions, implying that many PPL preparations investigated may have contained degradation products.
The technique of CsCl-density gradient centrifugation used to purify protein-polysaccharides was introduced by Franek and Dunstone (50). Their purification procedure proved to be very useful and CsCl-gradient centrifugation is today the purification method of choice for cartilage proteoglycans. In this technique strongly negatively charged macromolecules like protein-polysaccharides have a high buoyant density, and are obtained at the bottom of the centrifuge tube, while contaminating proteins which have few negative charges end up at the top. The technique was used to purify PPL (50) and it could be demonstrated that the purified preparation contained two components with different sedimentation rates in the analytical ultracentrifuge.

Dunstone and Franek (51) could demonstrate one 20-25S unit and one 75-85S unit in protein-polysaccharides prepared by CsCl-density gradient centrifugation. They also found a smaller unit of 6-7S in the preparation.
At that time, however, there were at least three explanations for the fact that different authors obtained different results and several fractions of proteoglycans - first the chance of proteolytic degradation during handling, second the mechanical treatment introduced high shear forces that could cause degradation of macromolecules (53) and third the different preparations were obtained with extraction methods differing in efficiency.
A major break through in the field of proteoglycan[1)] chemistry therefore came with the works of Hascall and Sajdera (52, 54, 55). They pointed out that the high speed homogenisation procedure generally used to extract cartilage pro-

---

1) The term proteoglycan was generally accepted with the work of Hascall and Sajdera (54, 55).

teoglycans caused degradation of the molecules due to the high shear forces obtained (52, 53). Sajdera and Hascall (52) found that from slices of nasal cartilage the proteoglycans could be isolated in very good yield (85 % of the total) by gently shaking the tissue in strong salt solutions like 3 M $MgCl_2$, 2 M $CaCl_2$ or 4 M guanidinium chloride. The proteoglycans were subsequently purified by CsCl gradient centrifugation in 0.4 M guanidinium chloride, to yield a preparation referred to as PPC (protein-polysaccharide complex) and which showed a bimodal distribution in the analytical ultracentrifuge, with one major fast sedimenting and one minor slow sedimenting component. In some elaborate experiments Hascall and Sajdera (54) showed that in 4 M guanidinium chloride only the slow sedimenting component could be demonstrated. By CsCl gradient centrifugation in 4 M guanidinium chloride they could subsequently separate the slow sedimenting component, which they called proteoglycan subunit (PGS), from a fraction rich in protein called glycoprotein link (GPL). They concluded that PPC contained aggregates of PGS and GPL, and demonstrated in some crucial experiments that GPL and PGS could indeed form aggregates. It was suggested that strong salt solutions like 4 M guanidinium chloride dissociated the aggregates present in vivo thereby facilitating extraction. Reaggregation was shown to occur whenever the salt concentration was lowered. Aggregation was dependent on pH and on intact S-S bonds of the molecules (54).

A two step purification method was presented (54). First the extract was centrifuged under "associative conditions" in a CsCl gradient in 0.4 M guanidinium chloride to obtain PPC. The PPC was again centrifuged in CsCl but this time under "dissociative conditions" - 4 M guanidinium chloride - to yield PGS and GPL. The isolated PGS had a very low protein content of about 6 % of dry weight and only some 8 % of the glycosaminoglycans were keratan sulfate. Subsequent work (55) showed that PGS is a polydisperse mixture of molecules with an average S_o of 25S and a M_w of $2.5 \pm 1.2 \times 10^6$. The limiting viscosity number was 141 ml/g. The very low α of 0.2 found for

the Staudiger equation suggested that PGS had a branched structure and it was further suggested that PGS had only one type of central proteoglycan core with several but a variable number of chondroitin sulfate chains attached to it (55). Thus the authors proposed polydispersity but not heterogeneity of PGS. The protein core was calculated to be of about 180,000 in molecular weight and each PGS carried some 90 chondroitin sulfate chains. The overall structure of the PGS was considered rather like that of a sphere.
When comparing PPL and PGS,Rosenberg et al. (56) showed that PPL-3 had a lower S_o value than PGS and that PPL-5 contained both aggregates and monomers. These authors suggested that the PGS molecules are formed from two subunits. However, one has to bear in mind that in the preparation procedure for PGS there is less chance for proteolysis than in the preparation of PPL.

An electron microscopic investigation on the structure of proteoglycans spread on a film of cytochrome (57) showed two forms of molecules from PPL, with a core of 3378 Å and 2033 Å, that had 30 and 20 chondroitin sulfate side chains respectively radiating out from a central protein core. PGS showed few short forms. On the basis of these pictures it was stated that two forms of proteoglycans were present, "monomer" and "doublet". Another investigation (58) using PGS (A1-D1 with the nomenclature of paper I) preparations from various types of cartilage have failed to demonstrate the long and short forms. In contrast the distribution of core lengths showed a Poisson distribution.
Tsiganos et al. (59) used the technique of Hascall and Sajdera (54) to isolate proteoglycans. They studied the gelchromatographic properties of the proteoglycans and could demonstrate that the Sepharose 2B profiles as well as glucuronic acid to protein ratios differed between proteoglycans of different buoyant density. These data were interpreted as indicating not only polydispersity but also heterogeneity of proteoglycans.

PRESENT INVESTIGATION

Since so many contradictory results on proteoglycan structure have been published using all sorts of preparations, the present work was initiated to try to bring some clarity to the structure and organisation of cartilage proteoglycans. The intentions were to make use of the dissociative extraction method combined with CsCl-density gradient centrifugation as described above, which permitted for the first time the isolation of proteoglycan molecules without exposure to the activity of the proteolytic enzymes known to be present in cartilage (36, 37). The proteoglycan preparations obtained would then better represent their native state and they should then be used to study their properties, organisation and structure - for instance regarding heterogeneity (differences in basic composition) or only polydispersity (the same basic composition but differences in proportion of building blocks). Further using cartilage as a model, the properties of proteoglycan from other tissues should be investigated.

Extraction and subfractionation of proteoglycans and variations in the protein core were studied in papers I and II. The distribution of polysaccharides along the protein core was investigated (papers II and III). The factors involved in aggregation of proteoglycans (IV) as well as the chemical characteristics of the aggregation (V, VI) were determined. A method was developed for the isolation of proteoglycans from tissues where these molecules are a minor component, and the proteoglycans obtained were compared to those of cartilage (VII).

Methodology

Tissues were extracted with 15 volumes (v/w) of 4 M guanidinium chloride, pH 5.8 (54) for 24 hours at 4^{o} C. With extracts or other preparations CsCl density gradients were performed under either "associative" (0.4 M guanidinium chloride) (54) or "dissociative" conditions (4 M guanidinium chloride)

(54), either to separate proteoglycans from proteins, or to fractionate proteoglycans according to buoyant density or ratio of polysaccharide to protein. The higher the proportion of charged polysaccharides, the higher the buoyant density. Gradients were established by centrifugation at 18° C for 48 hours at 34.000 rpm using an MSE 8 x 25 ml angle rotor. Centrifugation for longer times did not alter the distribution of material in the gradients.

For separation according to size gelchromatography on Sepharose or Sephadex gels was used. With few exceptions columns were eluted under associative conditions with 0.5 M sodium acetate, pH 7.0. V_0 and V_t were determined with proteoglycan aggregate and guanidinium chloride respectively. With some preparations a large proportion of the molecules appeared in the void volume, even on gels such as Sepharose 2B. Therefore in these cases no information on the size distribution of the molecules appearing in the void volume could be obtained. Column effluents were generally screened for chondroitin sulfate by determination of the uronic acid contents, for protein by colour yield in the Lowry procedure and for keratan sulfate by determination of neutral sugar contents. Procedures adopted for the Technicon autoanalyzer (60) were used.

Isolation and characterization of proteoglycans from tracheal cartilage (I).

In the first paper (I) the extraction and purification procedure is described and the fractions obtained are characterized by chemical and chromatographic procedures.

To increase the rate of extraction the cartilage was ground in a Wiley mill cooled with liquid nitrogen. Separate experiments showed that the extraction yield and physical properties - S_0 value and limiting viscosity - for proteoglycans prepared from cartilage that had been ground did not differ from those of proteoglycans that were isolated from tissue sliced with a SURFORM as described by Sajdera and Hascall (52). Guanidinium chloride being a strong

denaturating agent was preferred for extraction as a precaution against proteolytic attack on the proteoglycans during handling.
The yield of proteoglycans in the extracts was comparable to what is generally obtained from hyaline cartilage with the solvents used (52, 56, 59).

On associative density gradient centrifugation two fractions that contained aggregates with different polysaccharide to protein ratios were obtained. The subsequent dissociative centrifugation yielded a minor low buoyant density fraction corresponding to GPL, and several proteoglycan fractions free from contaminating proteins. The ratio of uronic acid (chondroitin sulfate) to protein varied from 3.02 to 0.41 with decreasing buoyant density of the fractions. In contrast the ratio of glucosamine (keratan sulfate) to protein was higher for the fractions with lower buoyant density. Comparatively large differences in the amino acid composition of the fractions were also observed. The relative contents of the amino acids known to occur near the chondroitin sulfate linkage region, namely glutamic acid, serine and glycine, decreased while the content of aspartic acid increased with decreasing buoyant density of the fraction. Proteoglycan fractions differing in chemical composition have also been obtained from pig laryngeal cartilage by Tsiganos et al. (59) using similar techniques.

The large differences in ratio of chondroitin sulfate to protein observed in the present investigation indicate, that the central protein core is substituted with chondroitin sulfate chains to a lower degree in fractions with lower buoyant density. In support of this assumption no difference was demonstrated for the size distribution of the chondroitin sulfate chains in the fractions (paper III). The large differences in amino acid composition suggest that the protein core exhibit heterogeneity. Thus proteoglycans may differ not only in terms of polysaccharide composition but also in terms of protein core. It may even be that the protein core is composed of subunits, one of which is rich in chondroitin sulfate chains and the other containing less or no polysaccharide. The data would then indicate that fractions with lower buoyant density would

contain a lower proportion of the chondroitin sulfate rich subunit. Indeed such a model is consistent with the results presented in papers III and VI.

This observation contradicts the suggestion of Hascall and Sajdera (55) that proteoglycans have only one type of protein core. However, others have also presented evidence that indicate heterogeneity of the proteoglycans (46, 47, 56, 59, 61, 62) and their data are supported by the results presented in paper I.

The fractions from the "dissociative" CsCl gradient gave different patterns on Sepharose 2B gelchromatography. The chromatograms obtained are very similar to those presented by Tsiganos et al. (59). The major bottom fraction was included in the gel. Since the major aggregate fraction obtained at the bottom of the tube in the associative centrifugation, was largely excluded from the same gel it appears that the proportion of aggregates in different proteoglycan preparations can be estimated from the ratio of excluded to included peaks on Sepharose 2B. The gelchromatogram for a fraction of intermediate buoyant density (D-2) in the dissociative gradient, however, showed a major excluded peak on Sepharose 2B indicating presence of aggregates. An explanation for this peak was obtained when the factors involved in aggregation were better characterized. As discussed in paper V hyaluronic acid, one of the factors involved in such aggregate formation, appears in fraction D-2 and under the associative conditions of the gelchromatography it most likely causes some of the molecules to aggregate and elute in the void volume. It is also shown in paper V that oligosaccharides that inhibit aggregation decrease the viscosity of this fraction. Furthermore Hardingham and Muir (63) have shown that hyaluronic acid is present in this part of a dissociative gradient.

Purification of link proteins (I)

In paper I is also described further subfractionation of the GPL fraction to yield 1) a low buoyant density proteoglycan fraction and 2) a protein fraction that contains very little hexosamine. A similar procedure has since been used

by Gregory (64) to yield a low buoyant density link protein fraction. On Sephadex G-200 gel chromatography this protein fraction gives an included peak that contained only protein, and an excluded peak that was rich in polysaccharides. It has thus been established that protein not substituted with polysaccharides is present in aggregates. The Sephadex G-200 included fraction contained the two proteins (unpublished observations) present in aggregates and GPL and which can be demonstrated by SDS-electrophoresis (65). Since Hascall and Sajdera (54) showed that aggregate formation was prevented after reduction and alkylation it is interesting to note that these proteins contain a relatively large amount of half cystine/cystine. Indirect evidence was obtained to indicate that these proteins could interact with proteoglycan monomers but could not form aggregates.

The proteoglycan protein core (II)

The evidence presented in paper I suggest that proteoglycans exist which differ in the composition of their protein core as well as in their polysaccharide composition.

The very high chondroitin sulfate content gives the proteoglycan a very large number of negatively charged groups as well as a large size. Since most fractionation methods make use of either size or charge or both, it is not possible to fractionate intact proteoglycans on the basis of properties of their protein core only, since these properties would be obscured by their polysaccharide part.

Hyaluronidase has been used by several authors to degrade the chondroitin sulfate chains of proteoglycans. Mathews and Lozaityte (33) used hyaluronidase digestion to study proteoglycan core-preparations. They suggested that the length of the protein core was not altered by the action of hyaluronidase and that chondroitin sulfate chains were attached via one or several attachment points. Similar results have later been obtained by others (34,66). Gregory et. al.(25) could isolate a protein core preparation of M_w 230,000, which contained

glucosamine. Indirect evidence obtained by degradation of proteoglycans with hyaluronidase provided evidence that keratan sulfate and chondroitin sulfate might be bound to the same protein core (44, 45).

No attempts, however, have been made to study the core of pure proteoglycans like those obtained with the Hascall and Sajdera method (54). Further it has been shown that proteoglycans isolated by procedures in which the dissociative CsCl-density gradient has been omitted, contain non-proteoglycan proteins (65, 67) that will complicate the interpretation of the results obtained.

It was therefore considered that removal of chondroitin sulfate side chains would accomplish 1) elimination of one of the major factors causing polydispersity and 2) leave a preparation representing protein cores. New additions to available techniques like the introduction of more porous gels such as agarose, for gelchromatography, in addition would make it possible to fractionate the proteoglycans and protein core-preparations according to size.

Monomer proteoglycans (D-1 of ref. 67) from tracheal cartilage were digested with highly purified hyaluronidase and chromatographed on Sepharose 6B to yield three protein peaks (H1, H2 and H3). The amino acid and hexosamine composition of the peaks differed. The larger peak contained a larger proportion of keratan sulfate than did the other two peaks.

Hascall and Riolo (68) used chondroitinase instead of hyaluronidase for the isolation of a proteoglycan core-preparation which showed polydispersity and which had an average molecular weight of 450,000 and a calculated protein-core weight of 200,000. This preparation also contained keratan sulfate. Fractions that had a higher relative glucosamine content also had a higher buoyant density on CsCl-gradient centrifugation but no separation into subfractions was demonstrated (68).

Alkaline degradation was used to degrade the proteoglycan fractions further, since O-glycosidic linkages to serine and threonine can be split by such treatment (26, 31). After alkaline treatment of the proteoglycan monomers

two protein peaks were observed on Sepharose 6B gel chromatography. In several reports the occurrence of two proteins after alkaline treatment of proteoglycans have been described (69, 70, 71, 72). However, since PPL type of molecules were used in these investigations, one has to take into account the presence of extraneous proteins that will complicate the chromatography patterns observed.

Conditions that caused a complete elimination of chondroitin sulfate chains was found to be 0.1 M LiOH for about 4 days in the cold. From the results discussed in paper II it appears that all chondroitin sulfate chains could be eliminated with alkali, since all the material that contained uronic acid appeared in one peak that did not coincide with any protein peak. Katsura and Davidson (73) in contrast have suggested that 20 % of the chondroitin sulfate is linked via glutamic acid to protein in an alkali stable linkage. More recent experiments performed by Simpson and Davidson (74) and Robinson and Hopewood (75), however, gave results in support of the idea that all chondroitin sulfate chains are linked to protein via alkali-labile linkages. When fractions of lower buoyant density were treated with alkali the proportion of the protein peak, first eluted from the Sepharose 6B column, was larger (unpublished observations), indicating heterogeneity of the protein cores. Further investigations on the effect of alkali on the three subfractions obtained on hyaluronidase digestion indicate further differences in composition. The large fraction (H1) contains a smaller proportion of substituted serine residues than do the other two fractions (76, unpublished results) indicative of a lower original chondroitin sulfate-content. This fraction (H1) also contained a relatively large proportion of "large molecular weight" keratan sulfate bound via a linkage stable under the alkaline conditions used. It (H1) in addition contained a smaller molecular size keratan sulfate which also was present in the other two fractions. The results obtained present evidence that the proteoglycan core is not one single entity; thus either several types of protein cores exist or the protein core may be

built from subunits linked via non-peptidic linkages. If such subunits exist at least one of these is likely to be enriched in keratan sulfate while others contain a larger proportion of chondroitin sulfate. The result is consistent with a proteoglycan-model where keratan sulfate is enriched in one part and chondroitin sulfate in another part of the core. Such a model has in fact been proposed (77, 78). Furthermore Serafini-Fracassini and collaborators studied degradation of proteoglycans with acetone-HCl (48) or triton x 100 (79) and suggested that proteoglycans are built from subunits.
It has also been argued (80) that a component may be present in rabbit ear cartilage,which can link subunits to form what would correspond to the proteoglycan monomers (PGS), but these results could not be reproduced with nasal or tracheal cartilage proteoglycans (unpublished observations).
Attempts were made to reveal the nature of the alkali-stable linkage between protein and keratan sulfate. Alkali-stable preparations of keratan sulfate and of glycopeptides from the keratan sulfate protein linkage region were found to contain a large proportion of glutamic acid/glutamine and proline, but no or small amounts of serine and threonine. Similar keratan sulfate fractions have been reported by others (68, 81),and Meyer (82) went as far as to suggest that skeletal keratan sulfate may be linked to glutamic acid or glutamine in the core protein, a type of linkage that in the light of the presented data appear to be plausible.

Distribution of chondroitin sulfate side chains in proteoglycans (III)

The organization of the chondroitin sulfate chains along the protein core has been studied using proteolysis. Thus Mathews (14, 83) found that chondroitin sulfate isolated from proteoglycans by trypsin digestion was of larger molecular size compared to that of preparations isolated after alkali treatment. Similar results were obtained by Buddecke et al. (11), Scheintal and Schubert (84) and Luscombe and Phelps (12). On the basis of these obser-

vations Anderson et al. (26) suggested that two chondroitin sulfate chains occurred in doublets and the chains were separated by a small peptide only. Mathews (14) found that trypsin and chymotrypsin digestion released chondroitin sulfate peptide fragments of similar size from proteoglycans from a variety of cartilages and used viscosity and osmotic measurements to indicate, that such fragments were chondroitin sulfate "doublets". The two chondroitin sulfate chains within such a doublet were thought to be separated by a short peptide and the doublets to be separated by a long peptide (14).

Attempts to split the proteoglycan core at the dehydroalanine (85), formed on β-elimination of chondroitin sulfate chains linked to serine, should theoretically yield two groups of peptides - one of a larger molecular size than the other. Experiments along this line, however, failed to demonstrate only two groups of peptides, instead a continuum of peptides of various sizes were observed (unpublished observations).

To investigate the structure of "doublets" further, chondroitin sulfate peptides were prepared by trypsin and chymotrypsin digestion of proteoglycans, as described by Mathews (14). The material was then subfractionated according to size by Sepharose 6B chromatography to yield 5 fractions with different elution volumes from the gel. Since nearly all of the material in the fractions was chondroitin sulfate it was concluded, that the chondroitin sulfate chains in the fractions were either of different size, or that a different number of chondroitin sulfate chains were attached per peptide molecule. Therefore, in order to reveal any difference in the length of the chondroitin sulfate chains, their distribution of molecular weights or sizes were determined according to Wasteson (86). No such difference between the major fractions could be demonstrated. It therefore seems likely, that there is a different number of chondroitin sulfate chains attached per peptide molecule in the fractions. That this was the case was shown by the use of ultracentrifugation to determine weight average molecular weights from 193,000 to 23,000 for the materials in the fractions. Since nearly all of the material in the fractions was chondroitin sulfate

with an average chain weight of about 20,000, it was concluded that chondroitin sulfate chains, in numbers that varied from 1 to about 10, were closely spaced along the peptide.

The molecular weight data and the limiting viscosity numbers of the fractions were used to establish constants for the Staudiger equation

$$[\eta] = K \cdot M^{\alpha}$$

The α value of 0.4 - 0.5 that was obtained indicates that the chondroitin sulfate peptides liberated by trypsin-chymotrypsin behaved like branched structures (87), as would be expected from a molecule that contains several large chains radiating out from a central core.

On the basis of chemical analysis Baker and Johnson (88) have suggested,that there are an average 5 chondroitin sulfate chains per peptide isolated by trypsin digestion of nasal cartilage proteoglycans. This figure is supported by the approximate average of 4 found in paper III.

All of the fractions had very similar amino acid composition with large proportions of serine, glutamic acid/glutamine and glycine. The similarity in amino acid composition for peptides substituted with chondroitin sulfate to such varying degrees may suggest that the sequence around the serine of the linkage region may be repetitive.

An interesting observation is that proteoglycans with very different polysaccharide to protein ratios,and thus with different buoyant densities in the CsCl gradient centrifugation,showed similar Sepharose 6B elution profiles of trypsin-chymotrypsin digests. This may suggest that the proteoglycan protein core is built from subunits, as discussed above, one rich in protein and one or several rich in chondroitin sulfate. According to such a model the proteoglycans of higher buoyant density would contain a larger proportion of the chondroitin sulfate rich subunits. Support for such a model was obtained when it was shown (paper VI) that the proteoglycan protein core contains a large protein domain with no chondroitin sulfate attached.

Electron microscopic investigations according to Rosenberg et al. (57) of the proteoglycans used in the present investigation gave the following results (58). Distance between side chains observed 114 A, with a very wide distribution of values. The number of side chains observed per molecule was 21. Since physical chemistry measurements indicate the presence of an average of 100 chondroitin sulfate chains per molecule (55) it may be,that each side chain observed on electron microscopy represent some 4-5 closely spaced chondroitin sulfate chains, a figure well in agreement with the average number of chondroitin sulfate chains observed per molecule of chondroitin sulfate peptide isolated from trypsin-chymotrypsin digested proteoglycans.

It appears that the chondroitin sulfate chains in tracheal and nasal cartilage proteoglycans are bound to protein in clusters which contain from 1 to several chains. The clusters are separated by peptides of variable length, as suggested by the electron microscopic pictures.

What is the explanation then, for the results others have obtained indicating that the chondroitin sulfate chains were bound in "doublets" to the peptide (11, 12, 14, 84)? Since the preparations, including those described in paper III, had similar chemical composition and gave similar viscosity data indicating structural similarity, the differences observed may be explained as follows.

While others obtained their data with unfractionated polydisperse materials that contained a substantial amount of chondroitin sulfate monomers, which gave the mixture an average molecular weight value that was too low, the data presented in paper III were obtained with materials fractionated according to size to give more homogeneous fractions, which will give more representative average molecular weight values.

Characteristics of aggregates from nasal and tracheal cartilage (IV)

The chemical data obtained with proteoglycan monomers and aggregates from tracheal and nasal cartilage are very similar not to say identical (54, paper

II). To discern whether or not the chemical similarity was also reflected in a similarity in physical properties, the S_0 values and limiting viscosity numbers were determined for tracheal and nasal proteoglycans. The monomer proteoglycans gave similar limiting viscosity numbers of 145 and 140 ml/g respectively. These figures agree well with those obtained by several authors for similar proteoglycan preparations like PPL-3 and PGS (37, 39, 51, 55, 56, 62). In the analytical ultracentrifuge the schlieren pattern of tracheal monomers showed a major component with an S_0 of 20S. Also a small somewhat faster sedimenting component that was not present in nasal proteoglycan monomers, which had an S_0 of 25S, was demonstrated. In contrast, however, the Sepharose 2B gelchromatograms of the two preparations were identical (unpublished observations). Several authors have obtained similar S_0 values for cartilage proteoglycans (43, 56, 89).

However, the limiting viscosity numbers for the preparations that contained the aggregates (A1) differed markedly between the two cartilages (220 ml/g for tracheal and 295 ml/g for nasal cartilage aggregates). The magnitude of the figure for nasal cartilage aggregates is well in agreement with similar figures for corresponding preparations like PPL, PPL-5 and PPC (39, 40, 43, 51, 89, 90).

Since the monomer proteoglycans from the two tissues were of similar size, it appears that the aggregates from nasal cartilage could be larger than those from tracheal cartilage, as could in fact be demonstrated by sedimentation velocity experiments. The aggregate preparations from the nasal and tracheal cartilage both showed a slow sedimenting component with similar S_0 values of 25S and 24S. The fast sedimenting major component constituted some 80 % of the proteoglycans from both tissues. The S_0 value, however, differed markedly in that tracheal aggregate showed an S_0 of 43S while those from nasal cartilage had an S_0 of 93S.

The S_0 value for nasal cartilage proteoglycan aggregates agree with those found in the literature for preparations, that are now known to contain both aggre-

gates and monomers (51, 54, 91). Using articular cartilage proteoglycans Rosenberg et al. (62) obtained two fast sedimenting components - one minor of 600S and one major aggregate of 56S. The extremely fast sedimenting component may, however, represent proteoglycan aggregate-collagen or protein complexes (unpublished observations). The lower S_0 values obtained by others (42, 90) may be explained by degradation of the molecules while being prepared.
It seems possible that the proportion of aggregate proteoglycans in different kinds of hyaline cartilage is constant as well as are the sizes of the monomers, while the sizes of the aggregates may differ. The results to be described offers an explanation for the mechanism of such differences.

The role of hyaluronic acid in aggregation (IV)

Gregory (64) provided evidence that two factors are required for aggregation. One of these are the link proteins, that can be prepared from GPL, (64, 65, 67) and the other, which has a relatively high buoyant density, could be the hyaluronic acid found in the middle portion of a dissociative CsCl gradient (63), and which in small amounts can aggregate proteoglycan monomers as shown by Hardingham and Muir (92). Such aggregates are, however, not stable under the conditions of ultracentrifugation (64, 92).
It is shown in paper IV that hyaluronic acid can form aggregates with both nasal and tracheal cartilage monomer proteoglycans. The reduced viscosity of the aggregates formed depend on 1) the relative amount of the hyaluronic acid, the optimal concentration being just less than 1 % of the weight of the aggregate, and 2) the size of the hyaluronic acid. The larger the hyaluronic acid the larger was the reduced viscosity of the aggregates formed. Further the optimal reduced viscosity of aggregates obtained with one of the standard hyaluronic acid preparations was very similar to that of a tracheal aggregate preparation, and as will be discussed below, tracheal aggregates indeed appear to contain a hyaluronic acid of the same size as this standard preparation. In other experiments it was also shown that proteoglycan protein core preparations, pre-

pared by chondroitinase ABC digestion of monomer proteoglycans, were able to interact with hyaluronic acid in a fashion similar to that of intact monomers, indicating that the chondroitin sulfate was not essential for aggregation.

Hyaluronic acid amounting to less than 0.8 % of the weight of the original aggregate, could be identified after mild proteolysis of aggregates with papain It appears that the enzyme removes the region of the proteoglycan protein core that contain the bound chondroitin sulfate chains, and leave behind the part of the core protein that interacts with the hyaluronic acid. Thus a hyaluronic acid protein complex could be isolated. It contained two proteins of molecular weights 40,000 and 65,000. The former most likely was one of the link proteins (64, 65) as suggested by its electrophoretic mobility, and the latter might derive from the proteoglycan core protein. The proteins could be separated from the hyaluronic acid under dissociative conditions and then under associative conditions be brought to aggregate again. Interestingly disulfide bridges known to be essential for aggregate formation (54) were also shown to be essential for the integrity of these proteins.

Graded proteolysis with papain was found to remove more protein from the hyaluronic acid till finally all peptides were lost. During such a procedure the elution volume of the hyaluronic acid labelled with peptide did not change, indicating that the elution volume of the hyaluronic acid was not changed by the proteins attached. The elution pattern for the labelled hyaluronic acid from tracheal aggregates was similar to that of the standard hyaluronic acid preparation, which produced aggregates of the same reduced viscosity as that of tracheal aggregates, while the hyaluronic acid-protein complex from nasal aggregates was larger. When chondroitinase ABC digestion was performed prior to the papain treatment, the Sepharose 2B elution volume of the labelled hyaluronic acid was more retarded indicating some degradation of the hyaluronic acid. It appears that the aggregating factor 1) can bind and protect proteins from proteolysis and 2) can be degraded by chondroitinase. Hyaluronic acid present in aggregates fulfil these requirements.

Evidence for the presence of aggregates in the native tracheal or nasal cartilage was obtained by direct papain treatment of the tissue. Such a treatment of either cartilage yielded solubilized hyaluronic acid-protein complexes with the same composition and size, as those complexes obtained from the aggregates respectively. Since the hyaluronic acid protein complex could be prepared from cartilage under the associative conditions of papain digestion, it is likely that aggregation occurs in vivo. Furthermore proteoglycans prepared by so called disruptive techniques, and thus never dissociated during extraction, contain a fast sedimenting component of aggregates (51).

The size of the hyaluronic acid probably determines the size of the aggregate. Thus through determination of the gel chromatographic behaviour of the hyaluronic acid, information on the dimensions of the aggregate could be obtained.

A procedure involving partial proteolysis of the tissue as described, may prove useful for studies of the size of aggregates in different tissues and in various pathological conditions, where the amount of material available is small.

Specificity of the interactions (V)

Proteoglycan cores prepared by chondroitinase ABC digestion of proteoglycan monomers (93) or intact proteoglycans, were used to study their interaction with hyaluronic acid. It was shown in paper IV that at an optimal concentration of 0.8 % (w/w) hyaluronic acid to proteoglycan core, some 70 % of the core molecules could interact with the hyaluronic acid. In order to obtain some information on the specificity of the interaction, oligosaccharides of defined sizes were isolated by gelchromatography of a hyaluronidase digested hyaluronic acid. The oligosaccharides were carefully characterized by gelchromatography, and by β-glucuronidase digestion that was found to liberate the calculated amount of glucuronic acid from each oligosaccharide. The ability of the oligosaccharides to bind to the proteoglycan monomers were tested in two ways.

1) By ability to inhibit interaction between proteoglycan core and hyaluronic

acid preparations. On Sepharose 2B chromatography,hyaluronic acid - proteoglycan core complexes appear in a void volume peak,while free cores appear in an included and hyaluronic acid oligosaccharides in a total volume peak. The absence of a void volume peak, then, demonstrate absence of aggregates and effective inhibition. It was shown that the smallest oligosaccharide capable of inhibiting the interaction was a hyaluronic acid decasaccharide,or a nonasaccharide with glucosamine in both reducing and nonreducing end. Chondroitin - the analogue of hyaluronic acid only differing in the position of the C-4 hydroxyl of the hexosamine - on the other hand was ineffective in preventing aggregation.

2) The interaction between hyaluronic acid and intact proteoglycan monomer was also studied in the viscosimeter as suggested by Hardingham and Muir (personal communication). The ability of oligosaccharides to inhibit the interaction,was measured as the ability to decrease the viscosity of mixtures of hyaluronic acid and proteoglycan monomers. Hyaluronic acid decasaccharide was again effective at low oligosaccharide concentration of the same magnitude as the hyaluronic acid concentration used. Large excess of hyaluronic acid octa-, hexa- or tetra-saccharide over hyaluronic acid,however,produced a slight decrease of viscosity in mixtures with proteoglycan monomers,probably via some unspecific effect.

Similar data using viscosity measurements have also been obtained with pig laryngeal proteoglycan monomers and our hyaluronic acid oligosaccharides (94). The data suggest that the site on the proteoglycan that interacts is large and protein in nature. It shows a very high specificity for regions of hyaluronic acid extending over some 40-50 Å.

In contrast to the results obtained with synthetic mixtures of hyaluronic acid and proteoglycan monomers,not even a large excess of hyaluronic acid dodeca- or decasaccharide had any effect on the viscosity of intact nasal proteoglycan aggregates. It should be remembered,that these aggregates still contain the link proteins and it appears as if the presence of these proteins stabilize the

aggregate. Similarly Gregory (64) observed that hyaluronic acid - proteoglycan aggregates were not stable in the ultracentrifuge,while aggregates containing link proteins were.

By gelchromatographic experiments it was shown that the link proteins also could combine with hyaluronic acid. As described in paper I,it is likely that the link proteins can also bind to the proteoglycan monomer, and they probably stabilize the aggregates via interactions both with hyaluronic acid and proteoglycans.

Characterization of the structures interacting with hyaluronic acid (VI)

Investigations of proteoglycan structure by electron microscopy (57) gave pictures,that suggested that one end of the proteoglycan monomer was attached to the factor causing proteoglycans to form aggregates. This aggregating factor appears to be hyaluronic acid (63, 92, paper IV),and the high specificity for the proteoglycan - hyaluronic acid interaction (94, paper V) suggests that the part of the proteoglycan involved is of protein nature. Furthermore elimination of the major polysaccharide (chondroitin sulfate) from the proteoglycans did not influence the aggregation (papers IV, V). More evidence,that a protein part of the proteoglycan monomer was involved in aggregation,were obtained with limited papain digestion of aggregates. On such treatment aggregates yielded a hyaluronic acid that contained bound proteins,which could be dissociated from the polysaccharide by dissociative agents (paper IV). It is possible that one of these proteins could be derived from the proteoglycan core and may represent the hyaluronic acid binding region of the proteoglycan. No hexosamines were demonstrated in these preparations (paper IV),which is further proof that the aggregation phenomenon represents protein to hyaluronic acid interactions.

The proteins obtained after the papain proteolysis of aggregates were, however, partly degraded by the enzyme and several peptides were joined only via disulfide bridges (paper IV).

In order to elucidate this point further (paper VI),nasal cartilage proteogly-

can aggregates were subjected to proteolysis with the more specific enzyme trypsin. Nasal proteoglycan aggregates were first briefly digested with chondroitinase to remove the chondroitin sulfate side chains, but leave the hyaluronic acid of the aggregate intact. The chondroitinase digestion was interrupted by the addition of trypsin free of chymotryptic activity and digestion was continued for various times. Sepharose 2B chromatograms of such digests showed a protein peak corresponding to the hyaluronic acid protein peak obtained after papain treatment of aggregates (paper IV). Contrary to the result obtained with papain the amount of protein in this peak did not decrease when digestion was continued, not even with more trypsin added. A limit tryptic digest was therefore made. The hyaluronic acid protein complex was prepared and chromatographed on Sephadex G-200 under dissociative conditions, in 4 M guanidinium chloride, to yield the hyaluronic acid void volume peak and two included protein peaks. The molecular weights of the two proteins were some 80-90,000 and 45,000 respectively. They most likely correspond to the 65,000 and 40,000 molecular weight proteins obtained after papain digestion of aggregates (paper IV), but in contrast their sizes were not much altered by reduction, indicating an intact primary structure. The procedure for the isolation of the proteins was repeated using aggregates, which were reconstituted from radioactive proteoglycan monomers mixed with non-radioactive link proteins and vice versa. It was demonstrated that the larger protein originated from the proteoglycan monomer, while the smaller protein derived from one of the link proteins.

Both of the two proteins, isolated from the trypsin digest, were able to recombine with the hyaluronic acid. Such recombination experiments suggested that the hyaluronic acid binding region of the proteoglycans may occupy a space of about 8 hyaluronic acid disaccharides, in agreement with the results obtained with intact core molecules (paper V). The composition of the two proteins showed marked differences and differed also from the overall composition of the proteoglycan monomers. It also appears that the larger protein contained some keratan sulfate, which represent some 5-10 % of the original keratan sul-

fate content of the aggregate. It is likely then, that some of the keratan sulfate molecules may be attached to the protein core localized to the hyaluronic acid binding region. These data support those discussed in paper IV and indicate that keratan sulfate may not be evenly distributed along the proteoglycan protein core. That chondroitin sulfate and keratan sulfate are bound to different regions of the protein core has been suggested (73, 74).

From the data presented it appears that the proteoglycan protein core, with a known molecular weight of about 200,000 (68), consists of a hyaluronic acid binding region built from more than a third of the amino acids of the proteoglycan core to form a structure most likely of globular nature. The other part of the proteoglycan core is the part to which the chondroitin sulfate chains are attached.

Eyring and Young (89) from studies of ORD and CD suggested that proteoglycans had no secondary structure. These authors could not demonstrate unfolding of the proteoglycan in 5 M guanidinium chloride and support of this result is the extreme stability towards different agents observed for the hyaluronic acid binding region.

Proteoglycans from aorta, cornea and sclera. Preparation and characterization (VII)

Cartilage contains a large proportion of proteoglycans, while the concentration of these substances is very low in tissues like aorta and sclera. Cornea is intermediate in this respect.

Knowledge about the structure and synthesis of cartilage proteoglycans is rapidly increasing. In contrast little work has been done on the structure and composition of the proteoglycans in the other tissues mentioned. The reason may be that these proteoglycans not only constitute a small proportion of the tissue, but they are also hard to isolate. Extraction with water or weak salt solutions give bad yields of proteoglycans, which cannot easily be separated from the large amount of collagen present in the extracts. However, some attempts have been

made to isolate proteoglycans from these tissues. Toole and Lowther (95) chromatographed urea extracts of bovine heart valves on ECTEOLA-cellulose in the presence of urea at elevated temperature, and Öbrink (96) used urea to extract proteoglycans from skin. Precipitation with cetylpyridinium chloride (97) or CsCl-density gradient centrifugation (98) has also been used to purify proteoglycans extracted with water or urea (96). However, especially at elevated temperatures urea is potentially a dangerous agent because of,1) degradation of the proteoglycans at the elevated temperature (unpublished observations), and 2) urea decompose to yield alkaline solutions and also form cyanate (99), which can cause 3) chemical modifications of the proteoglycans.

The introduction of the procedure of Sajdera and Hascall (52) employing 4 M guanidinium chloride for extraction,made it possible to extract proteoglycans in good yields from most tissues with little risk of degradation due to enzymatic attack during extraction. When this procedure was adopted to tissues like aorta, however, a large amount of collagen was coextracted and made impracticable the use of gradient centrifugation in CsCl for further purification of the proteoglycans. Furthermore the salt concentration of the extract could not be lowered, because then collagen and proteoglycans coprecipitated. Most fractionation procedures, however, depend on the solutions having a lower ionic strength than that of the guanidinium chloride extracts. A procedure was therefore developed to purify the proteoglycans in such extracts.

The guanidinium chloride was exchanged for 7 M urea buffered with Tris-HCl, by dialysis at low temperature (+4^{0} C). The collagen and the proteoglycans remained in solution since no precipitate was formed. The solution was then fractionated on DEAE-cellulose, eluted at low temperature with salt solutions in Tris-buffered 7 M urea,to yield 3 fractions, one of which contained the water soluble proteoglycans. The other fractions contained almost no glycosaminoglycans - that is proteoglycans - but contained all the collagen extracted. The purified water-soluble proteoglycans could then be fractionated by CsCl-gradient centrifugation or by other separation techniques.

The conditions used in the procedure virtually eliminate the risks involved, when urea is present. The low temperature drastically reduces the decomposition of the urea (99), and the buffer used prevent pH-changes and also take care of any cyanate formed (100).

Tracheal cartilage proteoglycans were prepared in good yield using the procedure described. Gelchromatographic and chemical investigations showed, that these proteoglycans were in all aspects similar to those prepared by the procedure used in paper I. Degradation or modification, thus, do not appear to have occurred during extraction and purification.

The procedure was therefore used to prepare proteoglycans from sclera, aorta and cornea in yields (80-85 %) as good as those obtained with cartilage. None of these proteoglycans contained hydroxyproline, indicating that collagen was not a contaminant.

The proteoglycans from these tissues had lower buoyant densities in CsCl-gradients, than observed for cartilage proteoglycans. They had higher protein contents of 20 % for aorta and sclera and 40 % of dry weight for cornea proteoglycans. Further the preparations from the three tissues contained a glycoprotein type of oligosaccharides not present in cartilage proteoglycans.

The amino acid composition of the different proteoglycans also differed markedly. The relative contents of serine - the amino acid involved in linkage of chondroitin sulfate or dermatan sulfate to protein - decreased in the order cartilage, aorta, sclera and cornea proteoglycans. This is the same order in which the polysaccharide contents of the proteoglycans decreased. In contrast the contents of aspartic acid/asparagine - the amino acid of the protein-carbohydrate linkage of glycoproteins - was high for all but the cartilage proteoglycans, which did not contain glycoprotein type of oligosaccharides. The gelchromatographic behavior of the preparations differed. All the proteoglycans were of smaller size compared to the cartilage monomer proteoglycans, and those from sclera also showed heterogeneity on gelchromatography. Buddecke et al.(11) obtained

a lower S_0 value of 1.5S for the portion of the proteoglycans from human aorta that could be water-extracted (30 %), compared to 9S for the nasal cartilage proteoglycans. The protein content of the aorta proteoglycans determined by these authors was about 20 %, similarly to what was found in paper VII.

From the results presented it appears that proteoglycans from different types of tissues show marked differences in physical and chemical properties, and it can be questioned whether one can use cartilage proteoglycans for model substance, when discussing relation of properties of tissues in relation to properties of proteoglycans.
The method described has later been successfully applied in a study of turnover of proteoglycans from some of the tissues discussed (101).

GENERAL CONCLUSIONS

Proteoglycans were isolated in good yields from different types of connective tissues with a new method. The composition and sizes of these proteoglycans differed markedly compared to cartilage proteoglycans. It is not unexpected, that proteoglycans from different sources may have different structures, depending on what the function of the particular connective tissue would be.

Proteoglycans were extracted from hyaline cartilages and fractionated by CsCl-gradient centrifugations. Proteoglycan aggregates could be dissociated by 4 M guanidinium chloride to yield a protein fraction as well as proteoglycan monomers, with a wide distribution of protein to polysaccharide ratios. The proteoglycan monomers were included on Sepharose 2B, while the aggregates were excluded.
The proteoglycan monomers after hyaluronidase digestion gave three fractions differing in size. They contained protein and keratan sulfate in different proportions. The largest fraction was richer in keratan sulfate than the two smaller fractions, which probably derived either from parts of the proteoglycan core more rich in chondroitin sulfate, or from proteoglycan molecules different from

those of the major fraction (H1). Keratan sulfate with a high content of glutamic acid/glutamine and proline could be isolated from the major fraction, indicating that this keratan sulfate may be linked to protein via glutamine or glutamic acid - a protein to carbohydrate linkage not previously demonstrated. Strong evidence is presented to show that the proteoglycan core is not a homogenous protein, but that either several different cores exist,or that one core is built from subunits of different composition and function.

Data to support the hypothesis, that proteoglycans are built from subunits, were obtained on degradation of proteoglycan aggregates and monomers with proteolytic enzymes. It was demonstrated that a part of the protein core, which constituted some 1/2 - 1/3 of the total core protein, had a tertiary structure and had the ability to interact with hyaluronic acid. The other part of the protein core contains the bound chondroitin sulfate chains, which are assumed to be distributed in clusters,of some 1-10 chondroitin sulfate chains,along the remaining 1/2 - 2/3 of the protein core. It may be that the length of this other part of the protein core is shorter in proteoglycans of lower buoyant density. Indeed proteoglycans with lower buoyant density are more retarded on Sepharose 2B columns (unpublished observations).

It was also shown that hyaluronic acid was present in the proteoglycan aggregates and it appears, that the proteoglycan monomers bind to hyaluronic acid via the particular "globular" protein part of the core to form aggregates. The presence of the link proteins in the aggregates seem to be essential for stable interaction. The binding was shown to be very specific for hyaluronic acid and require a length of 5 disaccharide units of the polymer. The core protein, when bound to hyaluronic acid, extend over a space of some 8-9 hyaluronic acid disaccharides, while in the aggregates the proteoglycan monomers are spaced at a much larger distance of some 50 hyaluronic acid disaccharides apart. Thus in the aggregate the domain of neighbouring proteoglycan monomers may overlap to some extent, and it may be, that the spacing is determined by the size of the poly-

saccharide chains attached to the proteoglycan protein core. Evidence is presented that the size of the hyaluronic acid may very well determine the size of the aggregate, and that this size may be different for different tissues.

TENTATIVE MODEL FOR THE STRUCTURE OF PROTEOGLYCAN AGGREGATES

When the contributions to the structure of the extracellular matrix of cartilage presented in this work are combined with earlier observations, the following tentative model of the molecular organization of one of its components can be presented (fig. 1).

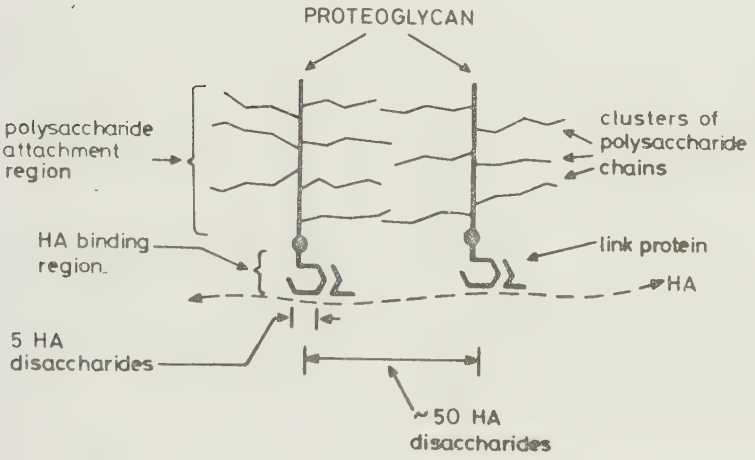

Fig. 1. Tentative model for the structure of proteoglycan aggregates.

ACKNOWLEDGEMENTS

This work has been made possible through the assistance of a number of persons to whom I am mostly grateful.

Professor Sven Gardell introduced me to the sticky field of connective tissue research and has since provided much helpful advice and constructive criticism. I appreciate his patience and generous assistance.

The collaboration of Vincent Hascall has been a great stimulus, and essential to many aspects of the work.

The many interesting discussions with drs. Lars-Ake Fransson, Inge Axelsson and the other members of the connective tissue group in Lund has been a great asset.

Much of the work has been performed by ms. Ewa Ljungberg and ms. Ulla Åkesson, who with patience and great ability dealt with all sorts of investigations.

Lillemor Hansson made it possible to get the words printed by providing excellent secreterial assistance.

This investigation has been supported with grants from the Swedish Medical Research Council, Konung Gustaf V:s 80-arsfond, The Medical Faculty, University of Lund, Svenska Sällskapet för medicinsk forskning, Alfred Österlunds stiftelse and Harald och Greta Jeansson's stiftelse.

REFERENCES

1. Mulder, G.J. (1838) Ann. Chem. 28, 328-330
2. Krukenberg, C.F.W. (1884) Z. Biol. 20, 307-326
3. Mörner, C.T. (1889) Scand. Archiv. Physiol. 1, 210-243
4. Schmiedenberg, O. (1891) Arch. Exp. Path. Pharmakol. 28, 355-404
5. Levene, P.A. (1925) Hexosamines and mucoproteins (Longmans, Green, London)
6. Meyer, K. and Rapport, M.M. (1950) Arch. Biochem. Biophys. 27, 287-293
7. Davidson, E.A. and Meyer, K. (1955) J. Amer. Chem. Soc. 77, 4796-4798
8. Meyer, K., Davidson, E., Linker, A. and Hoffman, P. (1956) Biochim. Biophys. Acta 21, 506-518
9. Linker, A., Hoffman, P., Meyer, K., Sampson, P. and Korn, E.D. (1960) J. Biol. Chem. 235, 3061-3065
10. Mathews, M.B. (1958) Nature 181, 421-422
11. Buddecke, E., Kröz, W. und Lanka, E. (1963) Hoppe-Seyler's Z. Physiol. Chem. 331, 196-218
12. Luscombe, M. and Phelps, C.F. (1967) Biochem. J. 103, 103-109
13. Wasteson, A. (1971) Biochem. J. 122, 477-485
14. Mathews, M.B. (1971) Biochem. J. 125, 37-46
15. Meyer, K. (1945) Advances in Protein Chemistry 2, 249-275
16. Balazs, E.A. and Jeanloz, R.W. (1965) The amino sugars Vol. II A, xiii-xiv (Academic Press, New York)
17. Meyer, K., Linker, A., Davidson, E.A. and Weissman, B. (1953) J. Biol. Chem. 205, 611-616
18. Bhavanandan, V.P. and Meyer, K. (1968) J. Biol. Chem. 243, 1052-1059
19. Hascall, V.C. and Riolo, R.L. (1972) J. Biol. Chem. 247, 4529-4538
20. Hammarsten, E. (1924) Biochem. Z. 144, 383-466
21. Jorpes, E. (1929) Biochem. Z. 204, 354-360
22. Meyer, K. and Smyth, E. (1937) J. Biol. Chem. 119, 507-510
23. Partridge, S.M. (1948) Biochem. J. 43, 387-397
24. Muir, H. (1958) Biochem. J. 69, 195-204
25. Gregory, J.D., Laurent, T.C. and Rodén, L. (1964) J. Biol. Chem. 239, 3312-3320

26. Anderson, B., Hoffman, P. and Meyer, K. (1965) J. Biol. Chem. 240, 156-167

27. Rodén, L. and Armand, G. (1966) J. Biol. Chem. 241, 65-70

28. Lindahl, U. and Rodén, L. (1966) J. Biol. Chem. 241, 2113-2119

29. Rodén, L. and Smith, R. (1966) J. Biol. Chem. 241, 5949-5954

30. Helting, T. and Rodén, L. (1968) Biochim. Biophys. Acta 170, 301-308

31. Anderson Bray, B., Lieberman, R. and Meyer, K. (1967) J. Biol. Chem. 242, 3373-3380

32. Shatton, J. and Schubert, M. (1954) J. Biol. Chem. 211, 565-573

33. Mathews, M.B. and Lozaityte, I. (1958) Arch. Biochem. Biophys. 74, 158-174

34. Partridge, S.M., Davis, H.F. and Adair, G.S. (1961) Biochem. J. 79, 15-26

35. Partridge, S.M. and Elsden, D.F. (1961) Biochem. J. 79, 26-32

36. Dingle, J.T. (1962) Proc. Roy. Soc. Med. 55, 109-111

37. Ali, S.Y. and Evans, L. (1973) Fed. Proc. 32, 1494-1498

38. Malawista, I. and Schubert, M. (1958) J. Biol. Chem. 230, 535-544

39. Gerber, B.R., Franklin, E.C. and Schubert, M. (1960) J. Biol. Chem. 235, 2870-2875

40. Pal, S. and Schubert, M. (1965) J. Biol. Chem. 240, 3245-3248

41. Pal, S., Doganges, P.T. and Schubert, M. (1966) J. Biol. Chem. 241, 4261-4266

42. Buddecke, E., Kröz, W. und Tittor, W. (1967) Hoppe-Seyler's Z. Physiol. Chem. 348, 651-664

43. Luscombe, M. and Phelps, C.F. (1967) Biochem. J. 102, 110-119

44. Tsiganos, C.P. and Muir, H. (1967) Biochem. J. 104, 26-28c

45. Heinegård, D. and Gardell, S. (1967) Biochim. Biophys. Acta 148, 164-171

46. Muir, H. and Jacobs, S. (1967) Biochem. J. 103, 367-374

47. Tsiganos, C.P. and Muir, H. (1969) Biochem. J. 113, 885-894

48. Serafini-Fracassini, A., Stimson, H. and Floreani, L. (1971) Biochem. J. 122, 101-106

49. Serafini-Fracassini, A., Peters, T.J. and Floreani, L. (1967) Biochem. J. 105, 569-575

50. Franek, M.D. and Dunstone, J.R. (1967) J. Biol. Chem. 242, 3460-3467

51. Dunstone, J.R. and Franek, M.D. (1969) J. Biol. Chem. 244, 3654-3659

52. Sajdera, S.W. and Hascall, V.C. (1969) J. Biol. Chem. 244, 77-87

53. Harrington, R.E. and Zimm, B.H. (1965) J. Phys. Chem. 69, 161-175

54. Hascall, V.C. and Sajdera, S.W. (1969) J. Biol. Chem. 244, 2384-2396

55. Hascall, V.C. and Sajdera, S.W. (1970) J. Biol. Chem. 245, 4920-4930

56. Rosenberg, L., Pal, S., Beale, R. and Schubert, M. (1970) J. Biol. Chem. 245, 4112-4122

57. Rosenberg, L., Hellman, W. and Kleinschmidt, A.K. (1970) J. Biol. Chem. 245, 4123-4130

58. Thyberg, J., Lohmander, S. and Heinegård, D., In manuscript

59. Tsiganos, C.P., Hardingham, T. and Muir, H. (1971) Biochim. Biophys.Acta 229, 529-534

60. Heinegård, D. (1973) Chemica Scripta 4, 199-201

61. Mashburn Jr, T.A. and Hoffman, P. (1971) J. Biol. Chem. 246, 6497-6506

62. Rosenberg, L., Pal, S. and Beale, R.J. (1973) J. Biol. Chem. 248, 3681-3690

63. Hardingham, T. and Muir, H. (1973) Biochemical Society Transactions 1, 282-284

64. Gregory, J.D. (1973) Biochem. J. 133, 383-386

65. Keiser, H., Schulman, H.J. and Sandson, J.I. (1972) Biochem. J. 126, 163-169

66. Cessi, C. and Bernardi, G. (1965) Structure and Function of Connective and Skeletal Tissue, pp. 152-156, (Butterworth's, London)

67. Heinegård, D. (1972) Biochim. Biophys. Acta 285, 181-192

68. Hascall, V.C. and Riolo, R.L. (1972) J. Biol. Chem. 247, 4529-4538

69. Marler, E. and Davidson, E.A. (1965) Proc. Natnl. Acad. Sci. U.S.A. 54, 648-656

70. Pedrini, V. (1969) J. Biol. Chem. 244, 1540-1546

71. Hoffman, P. and Mashburn Jr, T.A. (1967) J. Biol. Chem. 242, 3805-3809

72. Lyons, H. and Singer, J.A. (1971) J. Biol. Chem. 246, 277-285

73. Katsura, N. and Davidson, E.A. (1966) Biochim. Biophys. Acta 121, 128-134

74. Simpson, D.L. and Davidson, E.A. (1972) Biochemistry 11, 1856-1861

75. Robinson, C. and Hopwood, J.J. (1973) Biochem. J. 133, 457-470

77. Hranisavljevic, J., Simpson, D.L. and Davidson, E.A. (1972) Biochemistry 11, 2983-2990

78. Mayes, R.W., Mason, R.M. and Griffin, D.C. (1973) Biochem. J. 131, 541-553

79. Wells, P.J. and Serafini-Fracassini, A. (1973) Nature New Biol. 243, 266-268

80. Rokosova, B., Hanson, A.N. and Bentley, J.P. (1973) Biochim. Biophys. Acta 320, 442-452

81. Toda, N. and Seno, N. (1970) Biochim. Biophys. Acta 208, 227-235

82. Meyer, K. (1970) Chemistry and Molecular Biology of the Intercellular Matrix (Balazs, E.A. ed.) Vol. I, pp. 5-24 (Academic Press, London)

83. Mathews, M.B. (1956) Arch. Biochem. Biophys. 61, 367-377

84. Scheinthal, B.M. and Schubert, M. (1963) J. Biol. Chem. 238, 1935-1940

85. Patchornik, A. and Sokolovsky, M. (1962) Bull. Res. Counc. of Israel, 11A, 80-82

86. Wasteson, A. (1971) J. Chromatogr. 59, 87-97

87. Senti, F.R., Hellman, N.N., Ludwig, N.H., Babcock, G.E., Tobin, R., Glass, C.A. and Lamberts, B.L. (1955) J. Polymer. Sci. XVII, 527-546

88. Baker, A.J. and Johnson, A.H. (1973) Fed. Proc. 32, 676

89. Eyring, E.J. and Yang, J.T. (1968) J. Biol. Chem. 243, 1306-1311

90. Woodward, C.B., Hranisavljevic, J. and Davidson, E.A. (1972) Biochemistry 11, 1168-1176

91. Janado, M. (1971) J. Biochem. 69, 1123-1126

92. Hardingham, T. and Muir, H. (1972) Biochim. Biophys. Acta 279, 401-405

93. Hascall, V.C., Riolo, R.L., Hayward, J. and Reynolds, C.C. (1972) J. Biol. Chem. 247, 4521-4528

94. Hardingham, T. and Muir, H. (1973) Biochem. J. 135, 905-908

95. Toole, B.P. and Lowther, D.A. (1965) Biochim. Biophys. Acta 101, 364-366

96. Übrink, B. (1972) Biochim. Biophys. Acta 264, 354-361

97. Buddecke, E. und Schubert, M. (1961) Hoppe-Seyler's Z. Physiol. Chem. 325, 189-203

98. Franek, M.D. and Dunstone, J.R. (1968) Biochim. Biophys. Acta 165, 555-557

99. Hagel, P., Gerding, J.J.T., Fieggen, W. and Bloemendal, H. (1971) Biochim. Biophys. Acta 243, 366-373

100. Hirs, C.W.H. (1967) Methods in Enzymology (ed. Hirs), Vol. XI, pp.199-203, Academic Press, New York

101. Antonopoulos, C.A., Engfeldt, B., Gardell, S. and Heinegård, D. (1972) Scand. J. Lab. Clin. Invest. 29, suppl. 123, 4